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Interactions between the Nitrogen Signal Transduction Protein PII and *N*-Acetyl Glutamate Kinase in Organisms That Perform Oxygenic Photosynthesis

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PII, one of the most conserved signal transduction proteins, is believed to be a key player in the coordination of nitrogen assimilation and carbon metabolism in bacteria, archaea, and plants. However, the identity of PII receptors remains elusive, particularly in photosynthetic organisms. Here we used yeast two-hybrid approaches to identify new PII receptors and to explore the extent of conservation of PII signaling mechanisms between eubacteria and photosynthetic eukaryotes. Screening of *Synechococcus* sp. strain PCC 7942 libraries with PII as bait resulted in identification of *N*-acetyl glutamate kinase (NAGK), a key enzyme in the biosynthesis of arginine. The integrity of Ser49, a residue conserved in PII proteins from organisms that perform oxygenic photosynthesis, appears to be essential for NAGK binding. The effect of *glnB* mutations on NAGK activity is consistent with positive regulation of NAGK by PII. Phylogenetic and yeast two-hybrid analyses strongly suggest that there was conservation of the NAGK-PII regulatory interaction in the evolution of cyanobacteria and chloroplasts, providing insight into the function of eukaryotic PII-like proteins.

PII, one of the most conserved and widespread nitrogen signal transduction proteins, is an important player in the coordination of nitrogen assimilation and carbon metabolism (1, 8, 27). In the enteric system, two paralogous genes, *glnB* and *glnK*, encode PII proteins. Their receptors include converter enzymes (uridylyltranferase/uridylyl-removing enzyme or GlnD) and downstream targets (NtrB, the histidine kinase of the NtrB-NtrC two-component system, adenylyltransferase or GlnE, and the membrane-bound ammonium transporter AmtB). In cyanobacteria, a unique PII protein (referred to as GlnB), encoded by the *glnB* gene, is regulated by separate kinase and phosphatase activities (11); one of these activities, PphA, a PP2C-type phosphatase, has recently been identified in *Synechocystis* sp. strain PCC 6803 (18) as the PII phosphatase. In *Synechococcus* sp. strain PCC 7942, involvement of PII in the short-term ammonium inhibition of nitrate uptake has been well established (21), but direct protein-protein interactions between transport components and PII have not been reported.

Eukaryotic PII-encoding genes were first found in the chloroplast of the red alga *Porphyra purpurea* (31), and they seem to be present in a wide variety of higher plants, encoding proteins with high levels of homology to cyanobacterial PII proteins. Although PII from *Arabidopsis thaliana*, encoded by *GLB1*, has been identified and biochemically characterized, the physiological role of PII proteins in plants remains elusive (17, 26, 37). As it is in cyanobacteria, transcription of *GLB1* is regulated by light and carbon-nitrogen status (12, 17, 22), in agreement with a role in coordination of photosynthesis and nitrogen assimilation. Given the common evolutionary origin of PII proteins in organisms that perform oxygenic photosynthesis, it seems likely that mechanisms and components of the corresponding signal transduction systems may have been retained in cyanobacteria and plants.

In previous studies it has been shown that the yeast two-hybrid system very closely reflects functional interactions mediated by enterobacterial PII proteins (24, 25, 33). We are now using yeast two-hybrid approaches to identify PII receptors in cyanobacteria and to investigate the significance and extent of conservation of the interactions detected. We describe here identification and analysis of *N*-acetyl glutamate kinase (NAGK), a new PII receptor, and provide evidence suggesting that PII and NAGK are also interologues in photosynthetic eukaryotes.

MATERIALS AND METHODS

Strains and growth conditions. The strains and plasmids used in this work are listed in Table 1. Unless indicated otherwise below, cloning procedures were carried out with *Escherichia coli* DH5α by using standard techniques (34). The minimal medium used for the *E. coli* complementation test has been described previously (23). All constructs generated in this work were verified by automated dideoxy DNA sequencing. The yeast culture, transformation, and mating procedures used were procedures that have been described previously (3), unless indicated otherwise. For yeast two-hybrid interaction assays, derivatives of strain PJ696 carrying GAL4AD fusions were mated overnight with derivatives of strain Y187 carrying GAL4BD fusions in YPD liquid media in microtiter plates. Diploids were then analyzed for growth on different dropout media or for color development on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) indicator plates. The X-Gal overlay assay was performed as described previously (35). *Synechococcus* sp. strain PCC 7942 and derivative strains were grown photoautotrophically at 30°C with constant illumination (75 microeinsteins m^{-2} s^{-1}) provided by cool white fluorescent lights in BG-11 medium (containing nitrate), $BG11_0$ (lacking nitrogen), or $BG11_0$ supplemented with 5 mM NH_4Cl and 10 mM HEPES–NaOH (pH 8.0) (32). Media were supplemented with 10 mM $NaHCO_3$ and bubbled with a mixture of air and 1% CO_2.

Construction of plasmids. The sequences of the oligonucleotides used to construct plasmids are shown in Table 2. To construct pUAGC11 and pUAGC12, *glnB* sequences were amplified with primers GLNB-1F and GLNB-1R, cut with EcoRI and PstI, and cloned into pGAD424(+1) and pGBT9(+1),

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TABLE 1. Strains and plasmids.

Strain or plasmid	Genotype or relevant characteristics	Reference or source
E. coli DH5α	F^-ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1* λ^-	13a
E. coli HB101	F^- Δ(*gpt-proA*)*62 leuB6 glnV44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *rpsL20* (Str^r) *xyl-5 mtl-1 recA1*	34
E. coli MN8A	*argB2*	R. Cunin
S. cerevisiae Y187	MATα *ura3-52 his3-200 ade2-101 trp1-901 leu2-3, 112 gal4*Δ *inet*$^-$ *gal80*Δ *URA*::*GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ*	5b
S. cerevisiae PJ696	MAT**a** *ade2*Δ *trp1-901 leu2-3,112 ura3-52 his3-200 cyh*^{r} *can*^{r} *gal4*Δ *gal80*Δ *met2*$^-$ *GAL2*::*ADE2 GAL1*::*HIS3 GAL7*:*lacZ*	19a
Synechococcus sp. strain PCC7942		Pasteur Culture Collection
Synechococcus sp. strain MP2	Km^r derivative of strain PCCC 7942; kanamycin resistance cartridge inserted into the *glnB* gene	21
Synechococcus sp. strain MP2-A	Km^r Sm^r $GlnB^{S49A}$ derivative of MP2	21
pGAD424	Amp^r *LEU2*, GAL4(768–881)AD	3a
pGAD424(+1)	As pGAD424, with a different frame (+1)	32a
pGAD424(+2)	As pGAD424, with a different frame (+2)	32a
pGBT9	Amp^r *TRP1*, GAL4(1–147)BD	5a
pGBT9(+1)	As pGBT9, with a different frame (+1)	32a
pGBT9(+2)	As pGBT9, with a different frame (+2)	32a
pUAG161	GAL4AD:GlnB (*E. coli*)	25
pUAG162	GAL4BD:GlnB (*E. coli*)	25
pUAG181	GAL4AD:GlnK	33
pUAG182	GAL4BD:GlnK	33
pUAG211	GAL4AD:NtrB	24a
pUAG212	GAL4BD:NtrB	24a
pUAG401	GAL4AD:GlnD	This study
pUAG402	GAL4BD:GlnD	This study
pUAG421	GAL4AD:GlnE	This study
pUAG412	GAL4BD:GlnE	This study
pUAGC11	GAL4AD:PII (*Synechococcus*)	This study
pUAGC12	GAL4BD:PII (*Synechococcus*)	This study
pUAGC13	GAL4AD:PII^{S49A}	This study
pUAGC14	GAL4BD:PII^{S49A}	This study
pUAGC15	GAL4AD:PII^{S49D}	This study
pUAGC16	GAL4BD:PII^{S49D}	This study
pUAGC17	GAL4AD:PII^{S49E}	This study
pUAGC18	GAL4BD:PII^{S49E}	This study
pUAGC61	GAL4AD:NAGK (*Synechococcus*)	This study
pUAGC62	GAL4BD:NAGK (*Synechococcus*)	This study
pUAGC67	GAL4AD:$NAGK^{11–301}$ (*Synechococcus*)	This study
pUAGC69	GAL4AD:$NAGK^{74–301}$ (*Synechococcus*)	This study
pUAGC119	GAL4AD:PII (*A. thaliana*)	This study
pUAGC120	GAL4BD:PII (*A. thaliana*)	This study
pUAGC122	GAL4BD:NAGK (*A. thaliana*)	This study
pUAGC139	GAL4AD:NAGK (*P. purpurea*)	This study
pUAGC140	GAL4BD:NAGK (*P. purpurea*)	This study
pUAGC141	pTrc99A:*argB* (*Synechococcus*)	This study

respectively. Plasmid pUAGC62 was obtained by PCR amplification from pUAGC61 with TRANSGADGBT-1F and TRANSGADGBT-1R, followed by recombination cloning (20). To construct pUAG401 and pUAG402, *glnD* sequences were amplified with primers GLND-1F and GLND-1R, cut with BamHI, and cloned into pGAD424(+2) and pGBT9(+2), respectively. To construct pUAG421 and pUAG412, *glnE* sequences were amplified with primers GLNE-1F and GLNE-1R, cut with BamHI and PstI, and cloned into pGAD424(+2) and pGBT9(+2), respectively. To obtain plasmids pUAGC13 and pUAGC14, pUAGC11 sequences were first amplified with primers ACT-A and GLNB7942S49A-R and with primers GLNB7942S49A-F and ACT-B, respectively; the PCR products were then annealed and used as templates in new PCR with primers ACT-A and ACTB, and the products were cut with EcoRI and PstI and cloned into pGAD424(+1) and pGBT9(+1), respectively. Identical procedures were used to obtain plasmids pUAGC15 and pUAGC16 with primers GLNB7942S49D-R and GLNB7942S49D-F. Likewise, the same procedures were used to obtain plasmids pUAGC17 and pUAGC18 with primers GLNB7942S49E-R and GLNB7942S49E-F. To construct pUAGC119 and pUAGC120, *GLB1* sequences were amplified with primers GLB-AT-1F and GLB-AT-1R by using *Arabidopsis thaliana* cDNA as a template, cut with EcoRI and SalI, and cloned into pGAD424(+1) and pGBT9(+1), respectively. To construct pUAGC121 and pUAGC122, *ARGB* sequences were amplified with primers ARGB-AT-1F and ARGB-AT-1R by using *A. thaliana* cDNA as a template, cut with EcoRI and SalI, and cloned into pGAD424(+2) and pGBT9(+2), respectively. To construct pUAGC139 and pUAGC140, *argB* sequences were amplified with primers ARGB-PP-1F and ARGB-PP-1R by using *P. purpurea* total DNA as a template, cut with EcoRI and SalI, and cloned into pGAD424 and pGBT9, respectively. To construct pUAGC141, the *argB* gene from *Synechococcus* sp. strain PCC 7942 was amplified by PCR by using primers ARGBOV-1F and ARGBOV-1R and genomic DNA as a template. The PCR product was subjected to digestion with XhoI and then to partial digestion with NcoI. The resulting fragment was cloned into the NcoI-SalI sites of pTrc99A (Stratagene).

Construction and screening of *Synechococcus* sp. strain PCC 7942 Y2H libraries. Genomic DNA was partially digested with Sau3AI and treated essentially as described previously (33). After electrophoresis in preparative 0.8% agarose gels, portions containing DNA fragments ranging mainly from 400 to 1,100 bp long

TABLE 2. Oligonucleotides

Oligonucleotide	Sequence
ACT-A	5′ GAAGATACCCACCAAACCC 3′
ACT-B	5′ CAGTATCTACGATTAATAG 3′
GBT-1F	5′ GTGGAGACTGATATGCC 3′
GBT-1R	5′ TAAAACCTAAGAATCAC 3′
TRANSGADGBT-1F	5′ CGCACATCATCATCGGAAGAGAGTAGTAACAAAGGTCAAAGACAGTTCAC TGTATCGCCGAACCCAAAAAAAGAGATCG 3′
TRANSGADGBT-1R	5′ ATAACTTATTTAATAATAAAAATCATAAATCATAAGAAATTCG CCCGGAA TTAGCTTGGCGTTTTTCAGTATCTACGATTC 3′
TRANSP0-1	5′ TCTAGATCGATGAATTCGATATC 3′
TRANSP0-2	5′ GATATCGAATTCATCGATCTAGAGG 3′
GLND-1F	5′ CGGGATCCTCACCCTTTATCGTTTGG 3′
GLND-1R	5′ CGGGATCCTCACCCTTTATCGTTTGG 3′
GLNE-1F	5′ CGGGATCCATGAAGCCGCTCTCTTCACC 3′
GLNE-1R	5′ CGCTGCAGTCATTCTTCCACCAGC 3′
GLNB-1F	5′ GGCTTAAGGAGAATTCCCTTGAAGAAG 3′
GLNB-1R	5′ AACTGCAGTCGACGCTGACTTAGATTGCGTCG 3′
GLNB7942S49A-F	5′ CGCTATCGCGGCGCGGAATACACGGTT 3′
GLNB7942S49A-R	5′ AACCGTGTATTCCGCGCCGCGATAGCG 3′
GLNB7942S49D-F	5′ CGCTATCGCGGCGACGAATACACGGTT 3′
GLNB7942S49D-R	5′ AACCGTGTATTCGTCGCCGCGATAGCG 3′
GLNB7942S49E-F	5′ CGCTATCGCGGCGAGGAATACACGGTT 3′
GLNB7942S49E-R	5′ AACCGTGTATTCCTCGCCGCGATAGCG 3′
GLB-AT-1F	5′ TCTTCTGATTGAATTCCAGACTCG 3′
GLB-AT-1R	5′ TCTTGAGGTCGACTTGTTCCTAAGAC 3′
ARGB-AT-1F	5′ CACCGAATTCCAGAGTCGAGATTC 3′
ARGB-AT-1R	5′ CAACTTTGATGTCGACCCATTACAGC 3′
ARGB-PP-1F	5′ GCGAATTCATGTTGACAAACACAGAAAGGG 3′
ARGB-PP-1R	5′ GACGCGTCGACTTATACAACTAACATTGACC 3′
ARGBOV-1F	5′ GCCCGCCCATGGCTAGCGAGTTTATC 3′
ARGBOV-1R	5′ TGGGCCTCGAGTTAGCCCGAGCCAAC 3′

were recovered and separately cloned into vectors pGAD424, pGAD424(+1), and pGAD424(+2) to generate a minimum of 5×10^5 independent clones per primary library in *E. coli*. DNA fragments ranging mainly from 1,000 to 3,000 long were recovered and cloned into a 1:1:1 mixture of pGAD424, pGAD424(+1), and pGAD424(+2) to generate a minimum of 3×10^6 independent clones in *E. coli*. To generate Tsp509I libraries, partial digestion of genomic DNA with Tsp509I (Amersham Pharmacia Biotech) was performed in order to recover independent fragments in different size ranges. After electrophoresis in preparative 0.8% agarose gels, portions containing DNA fragments ranging mainly from 400 to 800 bp long and portions containing DNA fragments mainly from 800 to 2,000 bp long were separately recovered and cloned into a 1:1:1 mixture of EcoRI-digested pGAD424, pGAD424(+1), and pGAD424(+2). The Sau3AI and Tsp509I primary libraries were used to transform *Saccharomyces cerevisiae* PJ696 carrying pUAGC12. Transformants were selected on YNB minimal medium without Leu, Trp, and His but with 1 mM 3-amino-1,2,4-triazole. Small aliquots were also plated on YNB medium without Leu and Trp to determine the number of total transformants. Individual colonies from selection plates were replica plated onto YNB medium plates without Leu and Trp with the following modifications: (i) no His and 1 mM 3-amino-1,2,4-triazole; (ii) no Ade; and (iii) no modification. Plasmid DNA was extracted from clones that were able to grow on the three media (candidate clones) and used to transform electrocompetent HB101. Leu^+ transformants were selected in E-glucose medium and used as a source of DNA. Prey plasmids from candidate clones were rescued and used to separately cotransform PJ696 with the bait plasmid (pUAGC12) and the vector (pGBT9).

NAGK activity. NAGK activity was determined basically as described previously (13). The reaction mixtures contained 0.4 M NH_2OH-HCl, 20 mM $MgCl_2$, 20 mM ATP, 1 mM dithiothreitol, and 40 mM *N*-acetyl-L-glutamate. The mixtures were incubated for 1 h at 37°C, the reactions were stopped with 1 volume of a solution containing 5% $FeCl_3 \cdot 6H_2O$, 8% trichloroacetic acid, and 0.3 M HCl, and the absorbance at 540 nm was determined. The extinction coefficient used was 456 $M^{-1}cm^{-1}$. In situ NAGK activity was measured by using permeabilized *Synechococcus* sp. strain PCC 7942 cells. An amount of cells corresponding to 25 to 30 μg of chlorophyll was harvested, washed with 10 mM Tris-HCl (pH 7.2)–50 mM NaCl, resuspended in a buffer containing 10 mM Tris-HCl (pH 7.2), 50 mM NaCl, and 440 μg of mixed alkyl trimethyl ammonium bromide per ml, and assayed for NAGK activity as described above.

Multiple alignments and sequence comparisons. Sequences were obtained from the annotated genomes, and multiple alignments were created by using the Clustal X program.

Nucleotide sequence accession number. The accession number for the NAGK gene sequence is AY354518.

RESULTS AND DISCUSSION

Identification of NAGK by yeast two-hybrid screening of *Synechococcus* sp. strain PCC 7942 libraries by using PII as bait. In spite of considerable efforts to characterize PII from organisms that perform plant-like photosynthesis, the cellular components of PII signal transduction pathways in these organisms remain unknown. To approach this issue, we are using yeast two-hybrid strategies to search for proteins that interact with PII in a cyanobacterial model system for nitrogen regulation. To this end, genomic libraries were constructed by partial digestion of *Synechococcus* sp. strain PCC 7942 DNA with Sau3AI or Tsp509I, and approximately 2×10^6 Sau3AI clones and 2×10^7 Tsp509I clones were screened in *S. cerevisiae* for interaction with PII. Prey plasmids from candidate clones were rescued and used to separately cotransform preparations with bait and vector plasmids. Clones that activated the reporters GAL2:*ADE2* and GAL1:*HIS3* specifically with the GAL4BD:PII fusion were considered positive at this stage. A total of 52 of 200 positives clones, corresponding to one Tsp509I fragment and two Sau3AI fragments, contained *argB*-like sequences. These clones expressed the deduced full-length NAGK protein and two N-terminally truncated polypeptides ($NAGK^{11\text{-}301}$ and $NAGK^{74\text{-}301}$), indicating that the region C terminal to amino acid 74 provides the binding determinants for PII.

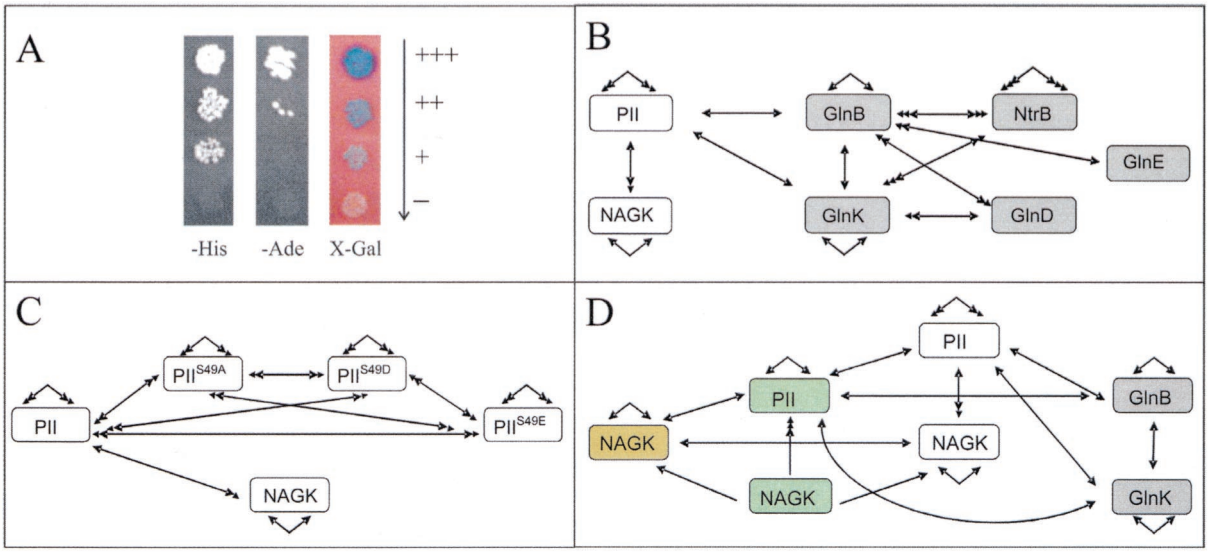

FIG. 1. (A) Levels of expression of GAL1:*HIS3*, GAL2:*ADE2*, and GAL1:*lacZ* in Y187/PJ696 diploids conferred by different fusion proteins. The relevant medium composition is indicated at the bottom. The photographs were taken 4 days after replica plating (−His and −Ade) or 4.5 h after addition of X-Gal (X-Gal). The vertical arrow indicates decreasing strength of the signals from interacting pairs. The plus and minus signs on the right indicate the relative strengths of the signals based on visual analysis. (B) Interactions involving PII proteins and receptors. (C) Effect of T-loop point mutations in Ser49 on interactions with NAGK. (D) Interactions among PII and/or NAGK fusion proteins. The arrows indicate the orientation of each Y2H interaction detected and point from the GAL4BD construct to the GAL4AD construct. The number of arrowheads indicates the relative strength of the interaction, as shown in panel A. The following color code is used: orange, *P. purpurea*; green, *A. thaliana*; white, *Synechococcus* sp. strain PCC 7942; and grey, *E. coli*.

NAGK is a member of the amino acid kinase family and catalyzes the second and obligatory step of arginine synthesis via *N*-acetyl glutamate in microorganisms and plants (5). In *E. coli*, the only organism for which the NAGK three-dimensional structure is available, the N-terminal domain binds *N*-acetyl glutamate and forms the dimer interface, while the smaller C-terminal domain binds ATP (7). Phenotypic complementation of an *E. coli argB* mutant with *Synechococcus* sp. strain PCC 7942 NAGK encoded in plasmid pUAGC141 was observed in medium lacking arginine, providing evidence of the functional equivalence of the *E. coli* and *Synechococcus* sp. strain PCC 7942 NAGK proteins.

Interaction analysis of bacterial PII proteins and receptors. To gain insight into the reliability and significance of the PII-NAGK interaction detected, we used the well-characterized enteric system and performed a yeast two-hybrid analysis with the two *E. coli* PII proteins (GlnB and GlnK), their three known receptors (NtrB, GlnD, and GlnE), and the *Synechococcus* sp. strain PCC 7942 PII protein and its prey (NAGK). This allowed us to obtain information concerning (i) the specificity of the interaction between the *Synechococcus* sp. strain PCC 7942 PII and NAGK proteins, (ii) heterologous recognition between PII proteins and between PII proteins and their receptors, and (iii) the extent of correlation between known functional interactions (including intersubunit interactions) and signals from the corresponding protein pairs. To minimize possible yeast two-hybrid artifacts, we analyzed all three reporter genes carried by host yeast strains and, for each pair of proteins, the two reciprocal combinations of fusion proteins (that is, each protein fused to each of the two GAL4 domains). Thus, we constructed additional GAL4AD and GAL4BD fusions to NAGK, PII, and other proteins of interest (see below). To determine the ability of two polypeptides to interact, the expression of reporters was analyzed on different selective or indicator media, and the results were categorized roughly into four groups, as follows: (i) no signal, (ii) significant but weak signals, (iii) strong signals, and (iv) very strong signals (Fig. 1A). A few of these combinations (NtrB paired with itself and with *E. coli* GlnB and GlnK) have been analyzed previously in *S. cerevisiae* Y190 (25, 33), and the strength of the signals was comparable to the strength of the signals detected here.

The results of the analysis of the two GAL4 domains fused to each of the seven PII proteins and receptors or of each domain by itself (vector plasmids) are summarized in Fig. 1B and Table 3. The strength and directionality of the signals strongly suggested that there was a true interaction between *Synechococcus* sp. strain PCC 7942 PII and NAGK. None of the constructs analyzed activated reporters by themselves, eliminating the possibility that there was direct transcriptional activation by individual fusion proteins. NAGK polypeptides (including the truncated derivatives $NAGK^{11-301}$ and $NAGK^{74-301}$ [data not shown]) failed to interact with any of the enteric PII proteins or PII receptors, ruling out the possibility that there was stickiness of the NAGK prey and confirming the specificity of the interactions detected in the screening analyses.

PII from *Synechococcus* sp. strain PCC 7942 did not give signals with any of the *E. coli* PII receptors, while significant interactions were observed between *E. coli* GlnB and each of the three enteric PII receptors and between GlnK and both NtrB and GlnD. No signals were detected between GlnK and GlnE, which is consistent with the finding that GlnK is not able

TABLE 3. Interactions among PII proteins and receptors from *Synechococcus* sp. strain PCC 7942 and *E. coli*: expression of reporters in PJ696/Y187 diploids

Organism	GAL4AD protein	Interactions with GAL4BD proteins[b]							
		Synechococcus sp. strain PCC 7942		*E. coli*					
		PII	NAGK	GlnB	GlnK	NtrB	GlnD	GlnE	NP[a]
Synechococcus sp. strain PCC 7942	PII	++	+	+	+	−	−	−	−
	NAGK	++	+	−	−	−	−	−	−
	GlnB	+	−	+	+	+++	++	++	−
	GlnK	+	−	+	+	+++	++	−	−
	NtrB	−	−	+++	++	+++	−	−	−
E. coli	GlnD	−	−	++	+	−	−	−	−
	GlnE	−	−	+	−	−	−	−	−
	NP	−	−	−	−	−	−	−	−

[a] NP, no protein fused to GAL4 domains.
[b] +, interaction equivalent to one arrowhead in Fig. 1; ++, interaction equivalent to two arrowheads in Fig. 1; +++, interaction equivalent to three arrowheads in Fig. 1; −, no interaction.

to effectively activate the adenylylation of glutamine synthetase (GS) by adenylyltransferase (2). Although it has been reported that *Synechococcus* sp. strain PCC 7942 PII can be uridylylated in vivo by *E. coli* GlnD (9), we did not detect signals between *Synechococcus* sp. strain PCC 7942 PII and any of the enteric PII receptors, suggesting that the interaction between the uridylyltranferase and *Synechococcus* sp. strain PCC 7942 PII is too weak or transient to be detected in yeast. Significant signals were obtained when NtrB (a dimer), each of the three PII proteins (trimers), and *Synechococcus* sp. strain PCC 7942 NAGK were paired with themselves, in agreement with previous reports on the dimeric structure of other NAGK proteins (7, 30).

Two-hybrid signals were also obtained when any of the PII proteins was paired with another PII protein, reflecting the reported ability of *E. coli* PII to form heterotrimers with both *Synechococcus* sp. strain PCC 7942 PII (10) and *E. coli* GlnK (38), in agreement with the view that heterologous heterotrimerization of PII proteins rather than NtrB targeting accounts for *Synechococcus* sp. strain PCC 7942 PII interference with PII-NtrB regulation in *E. coli* (9). When GlnE and GlnD were paired with themselves, no signals were detected, which is consistent with the monomeric structure of GlnE (19) and, to our knowledge, the lack of reports of quaternary structure for GlnD. In summary, although interpretation of negative data requires caution, these results illustrate the discrimination power of the analysis performed and the specificity of the interactions mediated by PII proteins.

Conserved Ser49 is required for interactions between *Synechococcus* sp. strain PCC 7942 PII and NAGK. In *Synechococcus* sp. strain PCC 7942 and other cyanobacterial PII proteins, Ser49 is phosphorylated in response to nitrogen-limiting conditions (11, 15, 21, 41). To investigate the effects of specific mutations in residue 49 on protein interactions with NAGK, we focused on mutations previously analyzed in *Synechococcus* sp. strain PCC 7942 (21) and constructed derivatives of the PII fusion proteins that might mimic either the phosphorylated (PII^{S49D} or PII^{S49E}) or unphosphorylated (PII^{S49A}) forms of the protein. The impact of these mutations on yeast two-hybrid interactions with PII receptors was analyzed. As shown in Fig. 1C and Table 4, none of the three mutant derivatives gave signals with NAGK. Since all three point mutation derivatives retained the ability to recognize all other PII derivatives, the possibility of negative effects on protein stability or intersubunit recognition can be excluded. Additional support for comparative folding of all PII derivatives constructed here came from the fact that all eight constructs gave signals with PipX (Table 4), another *Synechococcus* sp. strain PCC 7942 PII receptor whose characterization will be reported elsewhere. These findings are in line with genetic separation of *E. coli* PII interactions with receptors (19b), a phenomenon for which the yeast two-hybrid system has proven to be informative (24).

The finding that each of the three point mutations at position 49 knocked out yeast two-hybrid interactions with NAGK was surprising because it argued against the hypothesis that PII^{S49A} mimics the unphosphorylatyed state of the protein (21, 29, 40). In addition, these results highlight the importance of Ser49 for contact with NAGK, raising the possibility that this residue is directly involved in regulation of NAGK activity.

NAGK and PII proteins from cyanobacteria and eukaryotic photosynthetic organisms. To our knowledge, all PII receptors that have been characterized so far are proteins with limited taxonomic distributions. In contrast, NAGK proteins are

TABLE 4. Interactions among PII, PII receptors, and PII mutant derivatives from *Synechococcus* sp. strain PCC 7942

Protein	Interactions[b]						
	NAGK	PII	PipX	PII^{S49A}	PII^{S49D}	PII^{S49E}	NP[a]
NAGK	+	++	−	−	−	−	−
PII	+	++	++	++	++	++	−
PipX	−	+++	−	+++	+++	+++	−
PII^{S49A}	−	++	++	++	++	++	−
PII^{S49D}	−	++	++	++	++	++	−
PII^{S49E}	−	++	+++	++	++	++	−
NP	−	−	−	−	−	−	−

[a] NP, no protein fused to GA24 domains.
[b] +, interaction equivalent to one arrowhead in Fig. 1; ++, interaction equivalent to two arrowheads in Fig. 1; +++, interaction equivalent to three arrowheads in Fig. 1; −, no interaction.

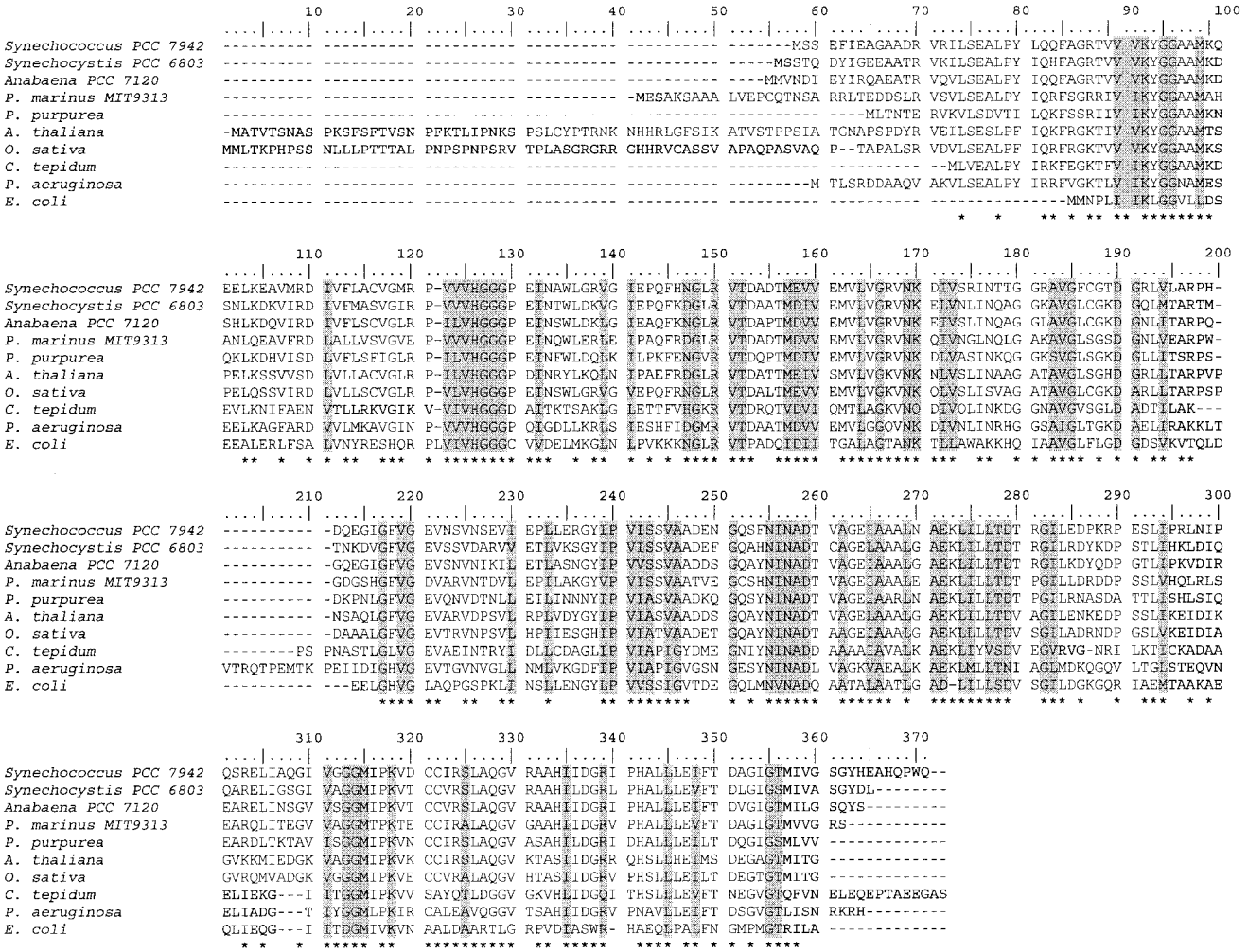

FIG. 2. Comparison of the deduced amino acid sequences of *Synechococcus* sp. strain PCC 7942 NAGK and representative bacterial and plant proteins. Regions conserved in all sequences are shaded. The asterisks indicate conservation in cyanobacterial and eukaryotic proteins. *P. marinus*, *Prochlorococcus marinus*; *O. sativa*, *Oryza sativa*; *C. tepidum*, *Chlorobium tepidum*; *P. aeruginosa*, *Pseudomonas aeruginosa*.

present in a wide variety of bacteria and plants, raising the question of whether the interaction between NAGK and PII is conserved in other taxonomic groups. The closest relatives of *Synechococcus* sp. strain PCC 7942 NAGK are other cyanobacterial proteins and eukaryotic proteins from algae and plants (Fig. 2). Oxygenic photosynthetic organisms synthesize arginine by the widespread cyclic pathway for ornithine production, and the activities of their NAGK proteins are feedback inhibited by arginine (5, 16). Not surprisingly, eukaryotic NAGK proteins (like PII proteins) are located in the chloroplast. All these facts strengthen the possibility that there is NAGK regulation by PII in photosynthetic eukaryotes. If this were the case, the predicted interacting determinants of PII proteins (that is, T loops) could also share structural features in these organisms. Interestingly, the presence of Ser at positions equivalent to the position of Ser49 of *Synechococcus* sp. strain PCC 7942 has already been reported for PII proteins from plants (17, 26).

An alignment of available Ser-containing T loops, along with a few other T loops from representative bacteria, is shown in Fig. 3. Ser49 is conserved in PII proteins from cyanobacteria, algae, and plants. In spite of the strict conservation of the phosphorylatable Ser49 residue in the cyanobacterium-chloroplast lineage, there are reports of PII proteins that do not seem to be phosphorylated in both cyanobacteria (28) and plants (36). It is worth noting that Tyr51 is also highly conserved in bacteria, although uridylylation or adenylylation at this position has been confirmed in only a few cases. In addition, PII proteins with Tyr51 appear to be regulated by phosphorylation at Ser49, irreversible proteolysis, or unknown mechanisms (14). Therefore, in spite of the strong conservation of Ser49 in cyanobacteria, algae, and plants, regulatory mechanisms other than phosphorylation are likely to operate in the cyanobacterium-chloroplast lineage.

In addition to Ser49, other features distinguish T loops in cyanobacteria (28), and some of them are common to the cyanobacterium-chloroplast lineage. With the exception of the red alga *Cyanidium caldarium*, the presence of Arg45 and Ser49 characterizes the cyanobacterium-chloroplast lineage, and the equivalent residues are Leu/Val45 and Ala49 in most

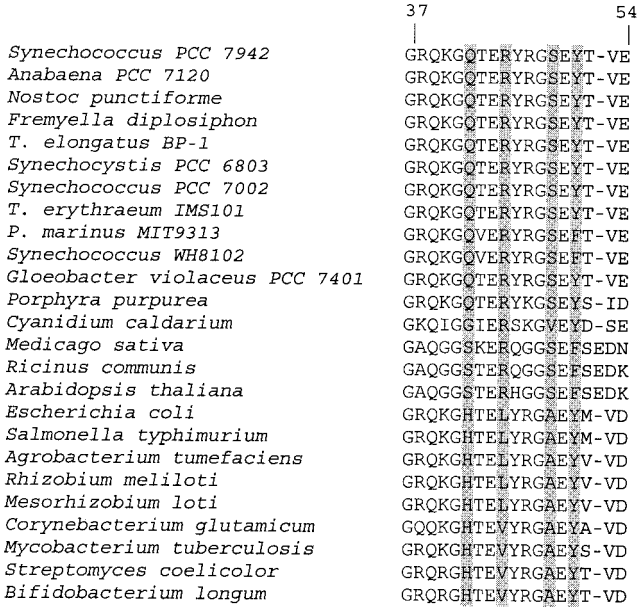

FIG. 3. Comparison of the deduced amino acid sequences of T loops from PII proteins of different organisms. The positions discussed in the text are shaded. The numbering is the numbering for *Synechococcus* sp. strain PCC 7942 PII. *T. elongatus*, *Trichodesmium elongatus*; *T. erythraeum*, *Trichodesmium erythraeum*; *P. marinus*, *Prochlorococcus marinus*.

other bacteria. In *E. coli* GlnB, point mutations L45R and A49P independently eliminate specific interactions with NtrB (24 and data not shown). A third position extends conservation to Gln42 (cyanobacteria and algae, again with the exception of *C. caldarium*), Ser42 (plants), and His42 (bacteria). Therefore, T loops from bacteria and the cyanobacterium-chloroplast lineage can be distinguished on the basis of these three positions.

Very few differences can be found among cyanobacterial T-loop sequences, suggesting that interactions with a combination of PII receptors have prevented divergence. The less extensive but still significant resemblance of T loops within the cyanobacterium-chloroplast lineage and, in particular, conservation of Ser49 can be taken as evidence of constraints imposed by interaction with a common receptor. On the basis of all data discussed so far, we hypothesize that the PII-NAGK interaction was present in the cyanobacterial ancestors (namely, oxygenic photosynthetic organisms).

Conserved interactions of PII and NAGK proteins in organisms that perform oxygenic photosynthesis. To obtain experimental support for conservation of the PII-NAGK interaction in oxygenic photosynthetic organisms, we generated additional GAL4AD and GAL4BD constructs for NAGK and PII proteins of *P. purpurea* and *A. thaliana* and carried out yeast two-hybrid analyses. The results, summarized in Fig. 1D and Table 5, were in complete agreement with our predictions. The NAGK-PII interaction was detected between *A. thaliana* proteins and between *A. thaliana* PII and *P. purpurea* NAGK, independent of the GAL4AD fusions used. Therefore, the finding that eukaryotic NAGK and PII proteins interact with each other supports our hypothesis that there was a Ser49-dependent PII-NAGK interaction in the cyanobacterial ancestors of chloroplasts.

Heterologous NAGK-PII interactions involving *Synechococcus* proteins were not detected, indicating that in spite of conservation of key T-loop residues, such as Ser49, divergence of additional interaction determinants between cyanobacterial and eukaryotic proteins prevented recognition. However, pairing of eukaryotic PII and NAGK proteins with themselves or with their orthologues gave signals in all cases in which the corresponding fusion proteins were appropriately expressed in yeast. *A. thaliana* PII gave signals with itself and with orthologous proteins from *E. coli* and *Synechococcus* sp. strain PCC 7942, suggesting the possibility that heterotrimers with the bacterial proteins were formed. Signals were also detected between NAGK proteins from *A. thaliana*, *Synechococcus* sp. strain PCC 7942, and *P. purpurea*, in agreement with their expected dimeric structures and the possibility that heterodimers between these proteins could be formed.

In the examples studied here, determinants involved in the oligomerization of PII and NAGK have diverged less than determinants involved in NAGK-PII recognition. This can be inferred by the type of signals obtained from heterologous pairs of *A. thaliana* and *Synechococcus* sp. strain PCC 7942 proteins, as follows: PII-PII, positive; NAGK-NAGK, positive;

TABLE 5. Interactions among PII and/or NAGK fusion proteins from *A. thaliana*, *P. purpurea*, *Synechococcus* sp. strain PCC 7942, and *E. coli*

Organism	Protein	Interactions[b]							
		E. coli		*A. thaliana*		*Synechococcus* sp. strain PCC 7942		*P. purpurea*	
		GlnB	GlnK	NAGK	PII	NAGK	PII	NAGK	NP[a]
E. coli	GlnB	+	+	−	+	−	+	−	−
	GlnK	+	+	−	+	−	+	−	−
A. thaliana	PII	+	+	+++	+	−	+	+	−
P. purpurea	NAGK	−	−	+	+	+	−	+	−
Synechococcus sp. strain PCC 7942	NAGK	−	−	+	−	+	++	+	−
	PII	+	+	−	+	+	++	−	−
	NP	−	−	−	−	−	−	−	−

[a] NP, no protein fused to GAL4 domains.
[b] +, interaction equivalent to one arrowhead in Fig. 1; ++, interaction equivalent to two arrowheads in Fig. 1; +++, interaction equivalent to three arrowheads in Fig. 1; −, no interaction.

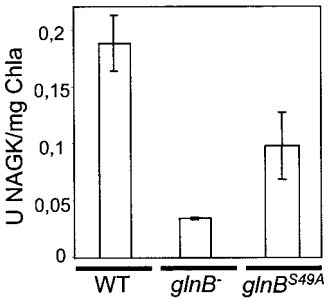

FIG. 4. Effect of PII on NAGK activity: NAGK assays with permeabilized cells of *Synechococcus* sp. strain PCC 7942 (WT), a PII knockout mutant (MP2) (*glnB*$^-$), and a derivative strain expressing the PII^{S49A} protein (MP2-A) (*glnB*^{S49A}). Cells were grown in ammonium-containing medium. The average values from three independent experiments are expressed in enzymatic units per milligram of chlorophyll (Chla).

and NAGK-PII, negative. The lack of heterologous recognition between NAGK and PII is consistent with the idea that in each organism, coevolution of NAGK and PII interacting determinants has been required to maintain a functional interaction between these proteins in the cyanobacterium-chloroplast lineage. In other words, for the PII-NAGK interacting determinants, both proteins have coevolved, so that their corresponding orthologues in the cyanobacterium-chloroplast lineage also interact, forming a group of interologues (39).

Is NAGK just a PII downstream target? In order to explore the physiological significance of the *Synechococcus* sp. strain PCC 7942 PII-NAGK interaction, we performed enzymatic assays using permeabilized cells with different genetic backgrounds (21). As shown in Fig. 4, the NAGK activity of the PII null mutant (PII$^-$) was four- to fivefold lower than the activity of the wild-type strain. In agreement with this, PII stimulation of NAGK activity was also observed in vitro (data not shown). Interestingly, the activity of the point mutation strain (PII^{S49A}) was two- to threefold lower, suggesting that in spite of the great impact of the S49A mutation on yeast two-hybrid assays for PII-NAGK interactions, PII^{S49A} proteins are still able to stimulate NAGK activity to some extent in *Synechococcus* sp. strain PCC 7942. However, the possibility that the PII and PII^{S49A} proteins could stimulate NAGK activity at additional levels of regulation cannot be excluded at present. Whatever the case, once again our results suggest that PII^{S49A} and unphosphorylated PII are not equivalent; that is, they differ at least in their interactions with NAGK.

The negative impact on *Synechococcus* sp. strain PCC 7942 PII-NAGK interactions of all three Ser49 substitutions used here supports the importance of this residue for PII-NAGK binding, raising the question of whether the conserved Ser49 residue of PII proteins from cyanobacteria and chloroplasts is directly involved in NAGK stimulation or rather plays a role in maintaining PII-NAGK contacts in vivo. In *Synechococcus* sp. strain PCC 7942 and other oxygenic photosynthetic organisms, NAGK activity appears to be regulated by two antagonistic signals of nitrogen sufficiency, arginine and (unphosphorylated) PII. It is worth noting that arginine is the amino acid with the highest nitrogen content and is frequently a key element of nitrogen storage compounds in photosynthetic organisms. It is tempting to speculate that the association of (unphosphorylated) PII and NAGK could also modulate PII interactions with other receptors, as has been proposed for the binding of GlnK to the ammonium transporter AmtB in proteobacteria (6). Since the levels of PII proteins and their receptors seem to be fine-tuned in order to avoid large changes in their relative levels (4), this hypothesis deserves further investigation.

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ERRATUM

Interactions between the Nitrogen Signal Transduction Protein PII and *N*-Acetyl Glutamate Kinase in Organisms That Perform Oxygenic Photosynthesis

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Volume 186, no. 11, p. 3346–3354, 2004. Page 3352: In the legend to Fig. 3, "*Trichodesmium elongatus*" should be "*Thermosynechococcus elongatus.*"